university of groningen

University Medical Center Groningen

University of Groningen

A novel NMR-based assay to measure circulating concentrations of branched-chain amino acids

Wolak-Dinsmore, Justyna; Gruppen, Eke G; Shalaurova, Irina; Matyus, Steven P; Grant, Russell P; Gegen, Ray; Bakker, Stephan J L; Otvos, James D; Connelly, Margery A; Dullaart, Robin P F

Published in:
Clinical biochemistry

DOI:
10.1016/j.clinbiochem.2018.02.001

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Wolak-Dinsmore, J., Gruppen, E. G., Shalaurova, I., Matyus, S. P., Grant, R. P., Gegen, R., Bakker, S. J. L., Otvos, J. D., Connelly, M. A., & Dullaart, R. P. F. (2018). A novel NMR-based assay to measure circulating concentrations of branched-chain amino acids: Elevation in subjects with type 2 diabetes mellitus and association with carotid intima media thickness. *Clinical biochemistry*, *54*, 92-99. https://doi.org/10.1016/j.clinbiochem.2018.02.001

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Clinical Biochemistry 54 (2018) 92–99

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

A novel NMR-based assay to measure circulating concentrations of branched-chain amino acids: Elevation in subjects with type 2 diabetes mellitus and association with carotid intima media thickness

Justyna Wolak-Dinsmore[a,*], Eke G. Gruppen[b,c], Irina Shalaurova[a], Steven P. Matyus[a], Russell P. Grant[a], Ray Gegen[a], Stephan J.L. Bakker[b,c], James D. Otvos[a], Margery A. Connelly[a,1], Robin P.F. Dullaart[b,1]

[a] Laboratory Corporation of America Holdings (LabCorp), Morrisville, Burlington, NC, USA
[b] Department of Endocrinology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[c] Department of Nephrology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

ARTICLE INFO

Keywords:
Branched-chain amino acids
Carotid intima media thickness
Metabolic syndrome
Nuclear magnetic resonance spectroscopy
Type 2 diabetes mellitus

ABSTRACT

Objectives: Plasma branched-chain amino acid (BCAA) levels, measured on nuclear magnetic resonance (NMR) metabolomics research platforms or by mass spectrometry, have been shown to be associated with type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). We developed a new test for quantification of BCAA on a clinical NMR analyzer and used this test to determine the clinical correlates of BCAA in 2 independent cohorts.
Design and methods: The performance of the NMR-based BCAA assay was evaluated. A method comparison study was performed with mass spectrometry (LC-MS/MS). Plasma BCAA were measured in the Insulin Resistance Atherosclerosis Study (IRAS, n = 1209; 376 T2DM subjects) and in a Groningen cohort (n = 123; 67 T2DM subjects). In addition, carotid intima media thickness (cIMT) was measured successfully in 119 subjects from the Groningen cohort.
Results: NMR-based BCAA assay results were linear over a range of concentrations. Coefficients of variation for inter- and intra-assay precision ranged from 1.8–6.0, 1.7–5.4, 4.4–9.1, and 8.8–21.3%, for total BCAA, valine, leucine, and isoleucine, respectively. BCAA quantified from the same samples using NMR and LC-MS/MS were highly correlated (R^2 = 0.97, 0.95 and 0.90 for valine, leucine and isoleucine). In both cohorts total and individual BCAA were elevated in T2DM (P = 0.01 to ≤0.001). Moreover, cIMT was associated with BCAA independent of age, sex, T2DM and metabolic syndrome (MetS) categorization or alternatively of individual MetS components.
Conclusions: BCAA levels, measured by NMR in the clinical laboratory, are elevated in T2DM and may be associated with cIMT, a proxy of subclinical atherosclerosis.

1. Introduction

The branched-chain amino acids (BCAA), valine, leucine and isoleucine, are essential amino acids that are not only required for protein synthesis but have been shown to regulate protein production, protein degradation and glucose metabolism. Decreased circulating concentrations of BCAA are associated with several pathological states including liver disease, early chronic kidney disease and all-cause mortality [1–4]. Increased BCAA, on the other hand, are associated with insulin resistance, type 2 diabetes mellitus (T2DM), coronary artery disease (CAD) and an altered microbiome [3,5–17]. While it has been hypothesized that increased circulation of BCAA may be a causal

Abbreviations: BCAA, branched chain amino acids; BMI, body mass index; CAD, coronary artery disease; cIMT, carotid intima-medial thickness; CLIA, Clinical Laboratory Improvement Amendments; CLSI, Clinical and Laboratory Standards Institute; CV%, coefficients of variation; CVD, cardiovascular disease; HDL, high density lipoprotein; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LDL, low density lipoprotein; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantitation; MetS, metabolic syndrome; NMR, nuclear magnetic resonance spectroscopy; ppm, parts per million; T2DM, type 2 diabetes mellitus; TG, triglycerides

* Corresponding author at: LabCorp, Morrisville, NC 27616, USA.
E-mail addresses: wolakdj@labcorp.com (J. Wolak-Dinsmore), e.g.gruppen@umcg.nl (E.G. Gruppen), shalaui@labcorp.com (I. Shalaurova), matyuss@labcorp.com (S.P. Matyus), grantr@labcorp.com (R.P. Grant), gegenr@labcorp.com (R. Gegen), s.j.l.bakker@umcg.nl (S.J.L. Bakker), otvosj@labcorp.com (J.D. Otvos), connem5@labcorp.com (M.A. Connelly), r.p.f.dullaart@umcg.nl (R.P.F. Dullaart).
[1] Contributed equally to the manuscript.

https://doi.org/10.1016/j.clinbiochem.2018.02.001
Received 22 September 2017; Received in revised form 22 December 2017; Accepted 3 February 2018
Available online 09 February 2018
0009-9120/ © 2018 The Authors. Published by Elsevier Inc. on behalf of The Canadian Society of Clinical Chemists. This is an open access article under the CC BY license (http://creativecommons.org/licenses/BY/4.0/).

factor in the development of insulin resistance and T2DM by contributing to overloading of mitochondria with lipid substrates, leading to mitochondrial stress and impaired insulin action [3,11], recent genetic evidence supports the concept that insulin resistance may in fact drive higher circulating fasting BCAA levels [18]. Consistent with these observations, BCAA may be predictive of diabetes development, and are responsive to therapeutic interventions that enhance insulin sensitivity [9,11,13,19,20]. In fact, fasting concentrations of BCAA were found to be elevated long before the onset of T2DM [9].

Measurement of circulating BCAA may thus be useful for determining early metabolic and organ dysfunction, well in advance of the development of chronic disease, thereby enabling preventative measures to potentially ameliorate disease progression. BCAA may also be useful in biomarker panels for discerning the metabolic pathways that are altered in individual patients, allowing for personalized treatment paradigms. The aim of the current study was to develop an assay for determining BCAA concentrations from NMR spectra collected for routine lipoprotein quantification on a clinical laboratory instrument. For this approach we developed a deconvolution model that takes into account all possible NMR signals from proteins and lipoprotein particles that overlap the signals from valine, leucine and isoleucine. With this newly developed NMR-based assay, we aimed to determine the extent to which NMR-measured BCAA levels are elevated in T2DM and metabolic syndrome (MetS). We also addressed the association of BCAA with carotid-intima media thickness (cIMT), a proxy of subclinical atherosclerosis and a well-established predictor of coronary heart disease and stroke [21,22].

2. Materials and methods

2.1. Materials and specimen collection

L-Isoleucine, L-leucine and L-valine were purchased from Sigma Aldrich (St. Louis, MO). Serum pools were prepared by identifying and pooling serum samples with high and low amino acid ranges from donor subjects (LabCorp, Morrisville, NC). This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), cleared by an Institutional Review Board and all donors signed consent forms.

2.2. NMR spectra acquisition and BCAA signal deconvolution analysis

NMR spectra were collected on a Vantera Clinical Analyzer (LabCorp, Raleigh, NC), a fully automated, high-throughput, 400 MHz proton (^{1}H) NMR platform. Serum or plasma samples were prepared on board the instrument by mixing 1:1 sample:NMR diluent (50 mM sodium phosphate, 120 mM KCl, 5 mM Na_2EDTA, 1 mM $CaCl_2$, pH 7.4) and automatically delivered to the flow probe in the spectrometer's homogeneous magnetic field [23]. NMR spectra were acquired in the same fashion as the *NMR LipoProfile* test [23,24]. Briefly, data acquisition on the Vantera was accomplished with water suppression using the WET solvent suppression technique [23,24]. The NMR data was acquired as 3 blocks of 4 scans for a total acquisition time of 48 s. Spectra were acquired with a sweep width of 4496.4 Hz and 9024 data points. The data was processed by zerofilling to 32 K points and multiplied by a Gaussian function to provide resolution enhancement prior to Fourier Transformation [23,24]. Two levels of controls were included at the beginning of each run of test samples for all experiments.

The methyl signals from the three BCAA in the ^{1}H NMR spectrum produce distinct patterns which can then be used for quantification (Fig. 1). These signals overlap with each other as well as with the methyl signals from the lipoprotein particles (Fig. 1). Spiking experiments with pure BCAA in serum helped identify the positions of the signature peaks of valine, leucine and isoleucine (Fig. 1 inset). The relative positions of the various signature peaks of isoleucine (doublet and a triplet), leucine (multiplet), and valine (two doublets) are distinct even within the many NMR signals from the other components in serum. In addition, experiments with pH titration helped us identify how these signals move in relation to each other as well as the reference peak (calcium EDTA). From these experiments we were able to derive mathematical equations that relate pH to position of the signature peaks within the NMR spectrum in order to orient the particular patterns of each BCAA so they could then be quantified individually for each patient. Therefore, for BCAA analysis, an optimized deconvolution algorithm was developed which simultaneously mathematically models the methyl signals from the lipoproteins, proteins and branched chain amino acids (between 0.718 and 1.02 ppm) in each NMR spectrum and quantifies valine, leucine and isoleucine. The BCAA concentrations were determined using non-negative linear least squares by first determining the signal areas of their well-characterized distinctive pattern of peaks and then multiplying by predetermined conversion factors (units = μM).

2.3. Assay performance testing

A Slide-A-Lyzer Dialysis 10 kDa molecular weight cutoff cassette (Thermo Scientific, Rockford, IL) was used to produce serum that was free of small molecules for determining the limits of blank (LOB). Serum pools containing low concentrations of valine, leucine or isoleucine (5 pools) were tested to determine the limits of detection (LOD) and quantitation (LOQ) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [25] as previously described [23]. Mean concentration and coefficients of variation (CV%) were calculated for each pool. Within-run and within-laboratory imprecision were determined based on CLSI guidelines using serum pools targeted at low, intermediate and high ranges for each of the BCAA [26]. Within-run (intra-assay) imprecision was determined by analyzing each of the pools on one day with 20 replicates. The same pools were analyzed for 20 days with two replicates twice per day (total n = 80) to evaluate the within-laboratory (inter-assay) imprecision. Consistent with CLSI guidelines [27], linearity was evaluated by comparing known spiked concentrations of the amino acids with expected concentrations. Linearity was tested in duplicate across the biological range from 0 to 600 μM by preparing dialyzed serum samples (as described previously) spiked with known amino acid concentrations.

2.4. Method comparison

Method comparison studies consistent with CLSI guidelines [28] were performed to compare BCAA quantification by NMR versus mass spectrometry (MS). Serum specimens were obtained from 21 donors and aliquots were frozen at −80 °C until the time of analysis. The same frozen serum samples were analyzed via Liquid Chromatography coupled to tandem mass spectrometry (LC-MS/MS, LabCorp, Burlington, NC) and NMR (LabCorp, Morrisville, NC). The LC-MS/MS method used external calibration with stable isotope dilution. Linear regression analysis and residuals plots were used to evaluate the correlation between the results from the two platforms.

2.5. Comparison of specimen collection tubes

Blood from 22 donors was drawn into three different tubes: Greiner Bio-One or LipoTube, BD Vacutainer serum tube (red top, no gel barrier) or K_2EDTA plasma tube. In order to expand the range of measured values, 5 specimens with low analyte concentration were diluted (≤50%) and 4 specimens with elevated analyte were spiked with the BCAA (≤10% by volume). A total of 31 specimens were tested for each analyte. Results for red-top serum and EDTA plasma tubes were compared to results for the LipoTube by linear regression and %bias was calculated.

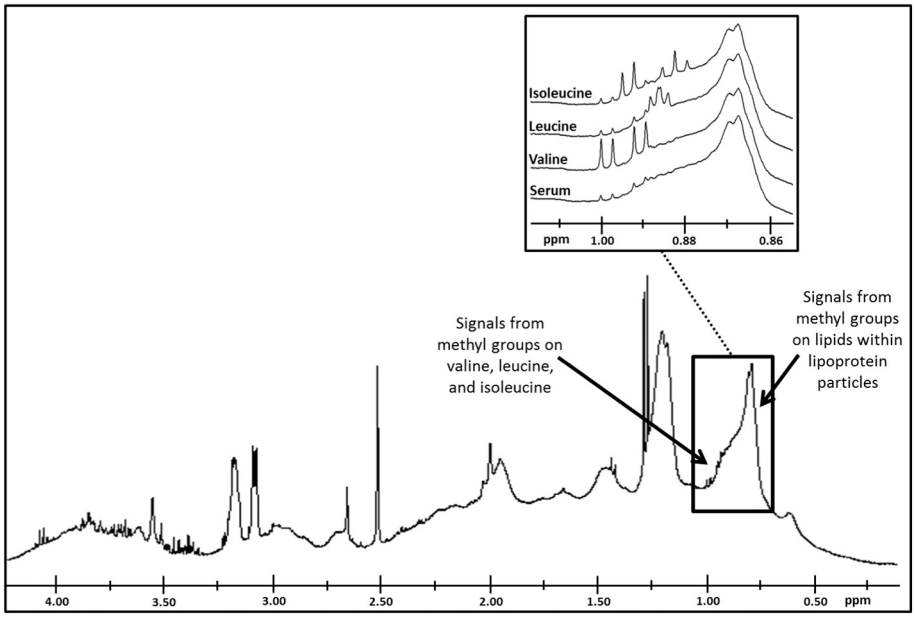

Fig. 1. NMR signal peaks for the branched chain amino acids (BCAA) in the *NMR LipoProfile* test spectrum of serum. The signals from the methyl groups on the BCAA overlap the signals that arise from the methyl groups on the lipid molecules contained within lipoprotein particles. Inset: Larger view of NMR spectra from serum spiked with the individual BCAA illustrating the relative positions of the various signature peaks of isoleucine (doublet and a triplet), leucine (multiplet), and valine (two doublets) compared to serum alone. ppm = parts per million.

2.6. Stability testing

Specimens from 20 donors drawn in LipoTubes were used to assess specimen/analyte stability. Samples were stored at 4 °C and aliquots were tested daily or tested after 4 months at −80 °C. Mean results for all donors were evaluated with acceptable differences falling within ± 10% of the day 0 (draw day) mean.

2.7. Cross-sectional studies in patients

2.7.1. Insulin resistance atherosclerosis study (IRAS)

The IRAS recruited 1625 participants from four clinical centers located in San Antonio, TX; San Luis Valley, CO; Oakland, CA; and Los Angeles, CA, between October 1992 and April 1994. Details of the study population, research methods and exclusion criteria have previously been published [29]. T2DM was defined as fasting glucose concentration ≥ 7.0 mmol/L and/or 2-h glucose concentration ≥ 11.1 mmol/L by a 75-g oral glucose tolerance test (OGTT) using World Health Organization criteria [30]. The use of insulin during a 5 year period before entry was an exclusion criterion. Demographic and socioeconomic information (e.g., age, sex, ethnicity), as well as lifestyle factors (e.g., smoking, alcohol consumption), were collected on standardized questionnaires by self-report [29,31]. 16% of participants were current smokers [32]. NMR spectra were acquired in 2000 from fasting plasma samples collected at baseline (1992–1994) (LipoScience, now LabCorp, Raleigh, NC) [30,33]. Quantification of BCAA was accomplished by reanalyzing the digitally stored NMR spectra using the newly developed BCAA software algorithm. The sample size of the current report was 1209 participants after exclusion of subjects with missing NMR data or diabetes status at baseline. The institutional review boards at each study site approved the study protocol, and all participants provided written informed consent.

2.7.2. Groningen cohort

T2DM and non-diabetic subjects, aged > 18 years, participated after written informed consent had been obtained. T2DM was diagnosed previously by primary care physicians based established criteria [34]. All participants were white. Insulin use was an exclusion criterion but the use of antihypertensive medication was allowed. Current smokers and subjects who used lipid lowering drugs were also excluded, as were subjects with a history of cardiovascular disease, chronic kidney disease (estimated glomerular filtration rate < 60 mL/min/1.73 m^2 and/or proteinuria), liver function abnormalities or thyroid dysfunction. NMR spectra were acquired in 2013 from fasting plasma samples collected between 2003 and 2004. After exclusion of subjects with missing BCAA results from 123 subjects were analyzed. The study protocol was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands.

2.8. Clinical procedures

Participants were asked to fast for 12 h and to abstain from alcohol and heavy exercise for 24 h. Body mass index (BMI in kg/m^2) was calculated as weight divided by height squared. Waist circumference was measured on the bare skin at the natural indentation between the 10th rib and the iliac crest. Blood pressure was measured using a standard mercury sphygmomanometer after participants were rested for 5 min (IRAS cohort) or 15 min (Groningen cohort).

2.9. Laboratory measurements

Venous EDTA-anticoagulated plasma and serum samples were collected after an overnight fast and stored at −80 °C until analysis. Glucose which was measured shortly after blood sampling using the glucose oxidase technique on automated instruments (IRAS: Yellow Springs glucose analyzer; YSI Inc., Yellow Springs, Ohio, USA; Groningen cohort: APEC glucose analyzer (APEC Inc., Danvers, MA)). In IRAS, lipids and lipoproteins were analyzed according to Lipid Research Clinic methodology [31]. In the Groningen cohort, lipids and lipoproteins were assayed by routine automated methods as described [34]. In the Groningen cohort, glycated hemoglobin (HbA1c) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands; normal range 27–43 mmol/mol).

2.10. Metabolic syndrome definition

MetS was defined according to NCEP-ATP III criteria. Three or more of the following criteria were required for categorization of subjects with MetS: waist circumference > 102 cm for men and > 88 cm for women; hypertension (blood pressure ≥ 130/85 mmHg or use of antihypertensive drugs); fasting plasma triglycerides ≥ 1.70 mmol/L; high density lipoprotein cholesterol (HDL-C) < 1.00 mmol/L for men and < 1.30 mmol/L for women; fasting plasma glucose ≥ 5.60 mmol/L.

Table 1
Within-laboratory (inter-assay) and within-run (intra-assay) imprecision for NMR-measured BCAA.

	Total BCAA (μM)			Valine (μM)			Leucine (μM)			Isoleucine (μM)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Within-lab[a]												
Mean	260.3	479.1	682.0	129.2	237.8	380.5	115.8	195.9	237.2	34.9	44.0	66.2
SD	15.7	15.5	12.3	5.8	7.4	8.9	9.7	11.6	11.2	7.4	6.2	6.5
CV (%)	6.0	3.2	1.8	4.5	3.1	2.3	8.4	5.9	4.7	21.3	14.1	9.8
Within-run[b]												
Mean	262.4	477.8	681.9	128.0	239.6	381.4	114.7	189.2	229.4	37.0	50.3	62.3
SD	11.5	15.6	14.6	6.9	6.8	6.4	10.4	8.8	10.0	5.8	6.0	5.5
CV (%)	4.4	3.3	2.1	5.4	2.9	1.7	9.1	4.6	4.4	15.8	11.9	8.8

BCAA, branched chain amino acids; CV, coefficients of variation; NMR, nuclear magnetic resonance; SD, standard deviation.
[a] Based on CLSI EP5-A2 tested using 3 controls, 2 runs per day in duplicate for 20 days (total n = 80).
[b] Based on 1 run of 20 tests.

2.11. Carotid intima media thickness (cIMT) measurement in the Groningen cohort

Of the 123 participants, 119 subjects had a successful cIMT measurement. CIMT was measured by ultrasonography in the supine position as described [34]. High-resolution B-mode ultrasound images were scanned (ACUSON 128 XP, Mountain View, CA, USA) with a 7.5 MHz linear array transducer. Three arterial wall segments of each carotid artery were imaged from a fixed lateral transducer angle at the far wall. The segments scanned were: the segment 1 cm proximal to the carotid dilatation (common carotid artery), the segment between the carotid dilatation and carotid flow divider (carotid bulb) and a 1 cm segment distal to the flow divider (internal carotid artery). The scans were recorded on S-VHS tape and analyzed off-line by an image analyst who was unaware of subject's characteristics. B-mode image analyses were digitized with a frame grabber (DT286 l; Data Translation Inc.; Marlboro, MA). The image analysis software was developed using an algorithm as described [21,34]. The mean cIMT of 6 carotid artery segments was calculated and used for analysis. At a mean cIMT of 0.80 mm, intersonographer variability was 0.05 mm, with an image analyst variability < 0.03 mm, corresponding to a total coefficient of variation (CV) < 7.5% [34].

2.12. Statistical analyses

Statistical analyses were performed using JMP version 12.1.0, SAS v9.4 (SAS Institute, Cary, NC), Analyze-it v3.90.1 (Analyze-it Software, Ltd., Leeds, UK), SSPS22 or GraphPad Prism 6.0. For the analytical validation studies, linear regression analyses were performed and Pearson correlation coefficients were determined for comparisons between continuous variables. For the epidemiological studies, data are expressed in mean ± SD (or SEM for figures) or in median (interquartile range). Skewed variables were natural log (Ln) transformed. Between-group differences in continuous variables were determined by unpaired *t*-tests. Between-group differences in dichotomous variables were determined by χ^2-analysis. Multivariable linear regression analyses were performed to determine the independent associations of BCAA, valine, leucine and isoleucine with diabetes status and the presence of MetS, as well as of cIMT with diabetes status, the presence of MetS, the individual MetS components and the plasma BCAA concentration. Two-sided *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical NMR BCAA assay performance

The NMR signals from the methyl groups on valine, leucine and isoleucine overlap each other as well as the methyl signals from the lipid molecules contained within the lipoprotein particles in the typical NMR LipoProfile test spectrum (Fig. 1). Therefore, an assay was developed that mathematically quantifies each individual BCAA in the context of each other as well as the lipoprotein concentrations in each sample (Fig. 1).

In order to show that we were able to accurately quantify individual BCAA despite the underlying NMR lipid methyl signals, NMR spectra were obtained from samples tested in the clinical laboratory. The spectra were analyzed using the BCAA assay (n = 3856 samples had results for both NMR BCAA and chemically-derived triglyceride; n = 3889 samples had results for both NMR BCAA and chemically-derived total cholesterol). The total cholesterol levels for these samples varied from 64 to 476 mg/dL and the triglyceride levels from 24 to 1153 mg/dL. Linear regression analysis was performed and Pearson correlation coefficients were calculated for the comparison of each BCAA with triglycerides or total cholesterol. None of the correlation coefficients reached statistical significance (Supplementary Table 2).

The analytical performance of the NMR-based BCAA assay was evaluated as per CLSI guidelines. The limit of blank (LOB) for valine, leucine and isoleucine were determined to be 13.9, 25.9 and 3.2 μM, while the analytical sensitivity or limit of detection (LOD) was calculated to be 22.6, 37.5 and 12.1 μM. The functional sensitivity or limit of quantitation (LOQ) for valine, leucine and isoleucine were determined to be 42.0, 38.0 and 15.0 μM, respectively. The LOQ for all three BCAA assays was below their reported normal reference intervals. Samples with three levels of each analyte (low, intermediate and high) were tested for intra- (within-run) and inter-assay (within-lab) precision. For total BCAA, valine, leucine, and isoleucine, the CVs for inter-assay and intra-assay precision were 1.8–6.0, 1.7–5.4, 4.4–9.1, and 8.8–21.3%, respectively (Table 1).

Linearity was demonstrated between 0 and 600 μM, well above the normal reference intervals, with correlation coefficients (R^2) of 0.99, 1.00 and 1.00 for valine, leucine and isoleucine, respectively (Supplementary Fig. 1A–C). Based on these data, the measuring or reportable ranges for valine, leucine and isoleucine are 42.0–600, 38.0–600 and 15.0–600 μmol/L, respectively.

A method comparison study was performed to compare BCAA quantification by NMR versus LC-MS/MS. Serum BCAA concentrations quantified using both platforms correlated well by linear regression with R^2 values of 0.97, 0.95 and 0.90 for valine, leucine and isoleucine, respectively (data not shown). Deming regression plots revealed R^2 values of 0.97, 0.94 and 0.90 for valine, leucine and isoleucine, respectively (Fig. 2A–C). The Bland-Altman plots for all 3 BCAA assays revealed that the residuals, while randomly dispersed, did show that there was a systematic bias toward higher results for valine and leucine and somewhat lower results for isoleucine when quantified by NMR compared to the MS assay (Fig. 2D–F).

Three types of specimen collection tubes (LipoTubes, red-top serum tubes and EDTA plasma tubes) were evaluated for their suitability in the

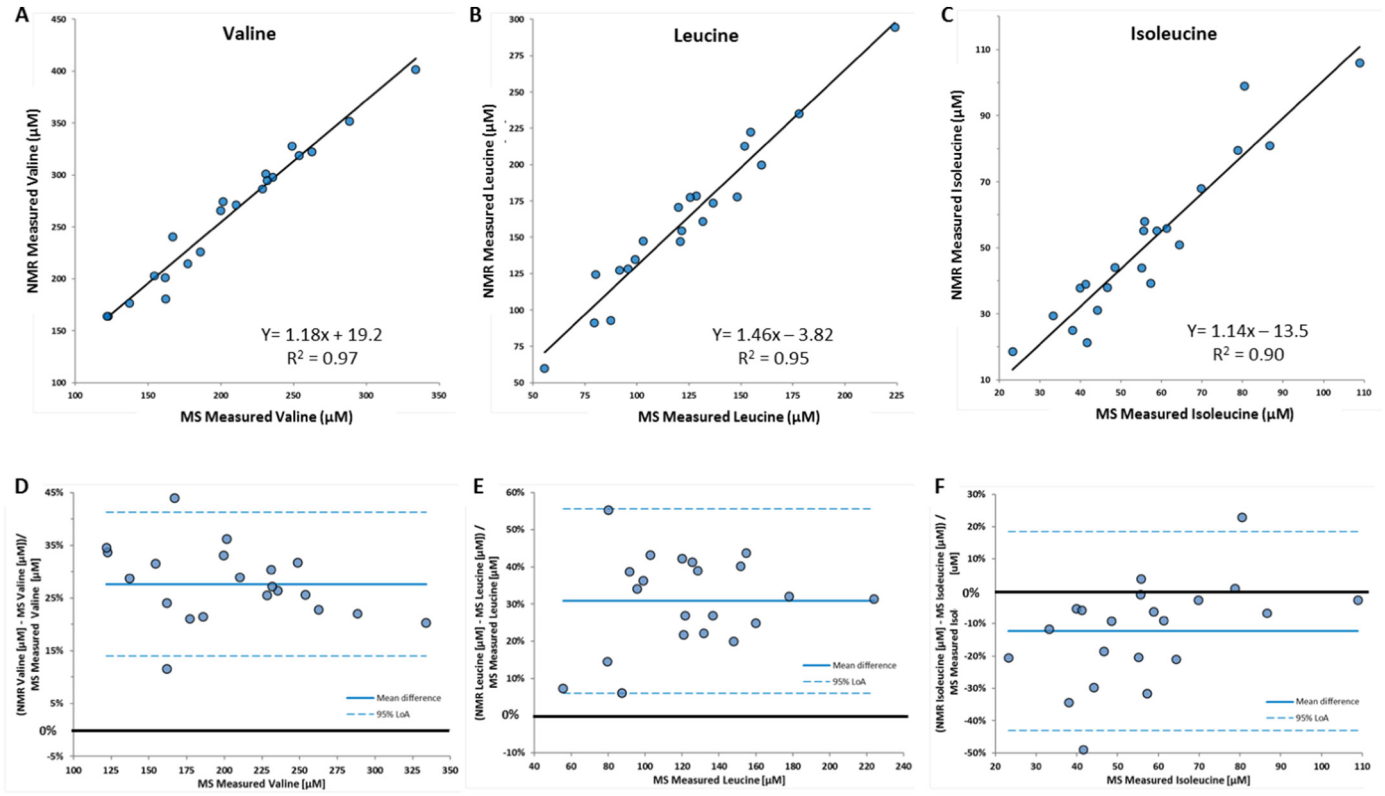

Fig. 2. Deming regression comparison between LC-MS/MS and NMR measured (A) valine, (B) leucine, and (C) isoleucine in serum samples (n = 21). Bland-Altman plots for (D) valine, (E) leucine, and (F) isoleucine assays. The limits of agreement (LoA) are depicted as dotted blue lines and the 0% bias is a solid black line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BCAA assay. Duplicate results for 31 specimens were compared between the various tube types. The results performed on specimens collected in LipoTubes were plotted against results from the red-top serum and EDTA plasma tubes and linear regression analyses were performed. Supplementary Table 1 summarizes the linear regression characteristics for the tube comparisons. Results indicate that there was minimal to no (< 5.0%) bias for valine, leucine and isoleucine in plain serum tubes versus serum collected in LipoTubes. However there were −6.3% and −13.6% biases for lower results for valine and leucine, respectively, in the EDTA plasma tubes versus LipoTubes.

The stability of each BCAA was evaluated in twenty serum samples stored refrigerated (2–8 °C) for up to 13 days or frozen at −80 °C for up to 4 months. Measurements were deemed acceptable if they were within 10% of the day 0 mean for the each individual analyte. Results demonstrated that valine and isoleucine were stable at 2–8 °C up to day 12. However, leucine was stable for 1 day at 2–8 °C. All 3 BCAA were stable when frozen at −80 °C for 4 months.

3.2. Relationship of circulating BCAA with T2DM and MetS

In IRAS, T2DM subjects were older compared to non-diabetic subjects (Table 2A). In the Groningen cohort, T2DM subjects were also older and were more likely to be men (Table 2B). In both cohorts, systolic blood pressure, BMI and fasting glucose were elevated in T2DM subjects. Waist circumference was not different according to diabetes status in IRAS but was higher in T2DM subjects in the Groningen cohort. In both cohorts, triglycerides were higher and HDL cholesterol was lower in T2DM subjects. Total cholesterol was lower in T2DM subjects from the Groningen cohort. In both cohorts, more T2DM subjects fulfilled the criteria for MetS (Table 2A and B). In both the IRAS and Groningen cohorts, total plasma BCAA, as well as valine, leucine and isoleucine levels separately were elevated in T2DM (all $P \leq 0.001$) (Table 2A and B). Total BCAA valine, leucine and isoleucine were also elevated in subjects with MetS compared to subjects without MetS, with the exception of leucine in IRAS (data not shown; $P < 0.001$). They were also elevated in men compared to women in each cohort (data not shown; $P < 0.001$ for each comparison).

Age- and sex-adjusted multivariable linear regression analysis demonstrated that total BCAA was found to be independently associated with diabetes status and the presence of MetS in each cohort (Table 3A and B). Likewise, valine was independently associated with the presence of T2DM and MetS. Leucine was independently associated with diabetes status in IRAS, and was associated with MetS in the Groningen cohort. Isoleucine was independently associated with T2DM and MetS in IRAS, and with MetS in the Groningen cohort.

We next evaluated the association between cIMT and BCAA taking account of diabetes status and MetS categorization in the Groningen cohort (n = 119). In crude analysis, mean cIMT was greater in T2DM subjects (n = 66; 0.884 ± 0.197 mm) vs. non-diabetic subjects (n = 53; 0.788 ± 0.153 mm, $P = 0.005$), as well as in subjects with MetS (n = 59; 0.892 ± 0.212 mm) vs. subjects without MetS (n = 60; 0.792 ± 0.137 mm, $P = 0.003$). In multivariable linear regression analysis taking account of age, sex, diabetes status and the presence of MetS, mean cIMT was associated with MetS, but not in the presence T2DM (Table 4, model 1; $R^2 = 0.306$, $P < 0.001$). In further analysis, the total BCAA concentration was positively associated with mean cIMT, independent of age and sex (Table 4, model 2; $R^2 = 0.324$, $P < 0.001$). Taking account of age and sex the association of cIMT with the total BCAA concentration remained statistically significant after additional adjustment for the use of sulfonylurea, metformin and antihypertensive medication (cf. Table 4 Model 2: $\beta = 0.230$, $P = 0.029$; data not shown). In multivariable analysis now including age, sex, diabetes status, presence of MetS and BCAA concentration, mean cIMT was associated with BCAA but not significantly with diabetes status and the presence of MetS (Table 4, model 3; $R^2 = 0.331$, $P < 0.001$). In alternative analysis including age, sex and the individual MetS

Table 2
Baseline clinical characteristics, metabolic control, plasma lipids and lipoproteins in the Insulin Resistance Atherosclerosis Study (IRAS) (A) and in the Groningen cohort (B).

A IRAS (n = 1209)	T2DM subjects (n = 376)	Non-diabetic subjects (n = 833)	*P*-value
Age (years)	57 ± 8	55 ± 8	< 0.0001
Sex, men (%)	180 (48)	358 (43)	0.13
Race			
Non-hispanic white (%)	128 (34)	333 (40)	0.04
Hispanic (%)	120 (32)	275 (33)	0.74
African American (%)	128 (34)	225 (27)	0.01
Metabolic syndrome (%)	271 (72)	292 (35)	< 0.001
Systolic blood pressure (mm Hg)	129 ± 18	122 ± 17	< 0.001
Diastolic blood pressure (mm Hg)	78 ± 10	78 ± 9	0.31
BMI (kg/m^2)	31.5 ± 5.6	28.4 ± 5.6	< 0.001
Waist circumference (cm)	94 ± 14	94 ± 13	0.43
Glucose (mmol/L)	9.8 ± 3.3	5.5 ± 0.6	< 0.001
Total cholesterol (mmol/L)	5.51 ± 1.12	5.45 ± 1.11	0.44
HDL-C (mmol/L)	1.05 ± 0.30	1.21 ± 0.39	< 0.001
Triglycerides (mmol/L)	1.77 (1.19–2.46)	1.24 (0.88–1.81)	< 0.001
Total BCAA (μM)	393 ± 77	337 ± 73	< 0.001
Valine (μM)	241 ± 41	210 ± 39	< 0.001
Leucine (μM)	102 ± 34	89 ± 32	< 0.001
Isoleucine (μM)	50 ± 19	39 ± 16	< 0.001

B Groningen cohort (n = 123)	T2DM subjects (n = 67)	Non-diabetic subjects (n = 56)	*P*-value
Age (years)	59 ± 9	54 ± 10	0.003
Sex (men/women)	41/26	23/33	0.041
Metabolic syndrome (%)	48 (72)	12 (21)	< 0.001
Systolic blood pressure (mm Hg)	145 ± 20	130 ± 20	< 0.001
Diastolic blood pressure (mm Hg)	87 ± 9	82 ± 12	< 0.001
BMI (kg/m^2)	29.0 ± 4.9	25.7 ± 4.1	< 0.001
Waist circumference (cm)	102 ± 13	87 ± 13	< 0.001
Glucose (mmol/L)	8.9 ± 2.3	5.6 ± 0.6	< 0.001
HbA1c (mmol/mol)	50 ± 7	34 ± 3	< 0.001
Total cholesterol (mmol/L)	5.34 ± 0.91	5.77 ± 0.97	0.014
HDL-C (mmol/L)	1.29 ± 0.39	1.54 ± 0.41	0.001
Triglycerides (mmol/L)	1.76 (1.20–2.17)	1.34 (0.88–1.91)	0.054
Total BCAA (μM)	499 ± 95	401 ± 87	< 0.001
Valine (μM)	278 ± 44	219 ± 41	< 0.001
Leucine (μM)	162 ± 37	139 ± 37	0.001
Isoleucine (μM)	59 ± 25	43 ± 19	< 0.001

Data in means ± SD or in medians (interquartile ranges). BCAA, branched chain amino acids; BMI, body mass index; HDL-C, high density lipoprotein cholesterol; T2DM, type 2 diabetes mellitus.

components the total BCAA concentration was also independently associated with cIMT (Table 4, model 4; $R^2 = 0.375$, $P < 0.001$).

4. Discussion

Our study is the first to show that accurate concentrations of plasma or serum valine, leucine and isoleucine, can be obtained from the same NMR spectra that is acquired for determining lipoprotein particle concentrations on a clinical NMR analyzer, without the need for suppression of the protein and lipoprotein signals [23,24]. Mass spectrometric assays have been developed that quantify BCAA both in the clinical and research settings. Additionally, BCAA concentrations have been estimated from high-volume NMR metabolomics research platforms where the spectra were collected using conditions in which the

Table 3
Multivariable linear regression analysis demonstrating associations of plasma total BCAA, valine, leucine and isoleucine with T2DM and MetS in the Insulin Resistance Atherosclerosis Study (IRAS) (A) and in the Groningen cohort (B).

	BCAA		Valine		Leucine		Isoleucine	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
A IRAS (n = 1209)								
Age	−0.015	< 0.001	−0.016	< 0.001	−0.012	0.0003	−0.007	0.030
Sex (men/women)	0.570	< 0.001	0.560	< 0.001	0.404	< 0.0001	0.425	< 0.001
T2DM (yes/no)	0.604	< 0.001	0.600	< 0.001	0.382	< 0.0001	0.517	< 0.001
MetS (yes/no)	0.297	< 0.001	0.384	< 0.001	0.037	0.53	0.323	< 0.001
B Groningen cohort (n = 123)								
Age	−0.038	0.60	−0.020	0.77	0.014	0.86	−0.139	0.097
Sex (men/women)	0.353	< 0.001	0.295	< 0.001	0.380	< 0.001	0.273	0.001
T2DM (yes/no)	0.216	0.010	0.349	< 0.001	0.011	0.90	0.159	0.099
MetS (yes/no)	0.395	< 0.001	0.339	< 0.001	0.409	< 0.001	0.314	0.001

β: standardized regression coefficient. All analyses are adjusted for age and sex. The data from IRAS are also adjusted for race. BCAA, branched chain amino acids; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus.

Table 4
Multivariable linear regression analysis demonstrating the association of mean carotid intima media thickness with diabetes status, presence of MetS and plasma branched chain amino acid (BCAA) concentration in the Groningen cohort (119 subjects; 66 subjects with T2DM and in 53 subjects without T2DM; 59 subjects with MetS and 60 subjects without MetS).

	Model 1		Model 2		Model 3		Model 4	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Age	0.387	< 0.001	0.404	< 0.001	0.395	< 0.001	0.314	< 0.001
Sex (men vs. women)	0.232	< 0.001	0.129	0.13	0.154	0.084	0.144	0.098
T2DM (yes/no)	0.020	0.83			−0.025	0.79		
MetS (yes/no)	0.193	0.035			0.107	0.27		
Elevated glucose							0.041	0.62
Elevated blood pressure							0.200	0.019
Enlarged waist							0.039	0.67
Elevated triglycerides							0.055	0.62
low HDL cholesterol							−0.135	0.12
BCAA			0.263	0.002	0.212	0.043	0.217	0.038

β: standardized regression coefficient. BCAA, branched chain amino acids; cIMT, carotid intima-media thickness; HDL, high density lipoproteins; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus.
Model 1: adjusted for age, sex, diabetes status and the presence of MetS ($R^2 = 0.306$).
Model 2: adjusted for age, sex and BCAA concentration ($R^2 = 0.324$).
Model 3: adjusted for age, sex, diabetes status, the presence of MetS and BCAA concentration ($R^2 = 0.331$).
Model 4: adjusted for age, sex, diabetes status, individual MetS components and BCAA concentration ($R^2 = 0.375$).

NMR signals from the proteins and lipoproteins were suppressed; often called Car-Purcell-Meiboom-Gill (CPMG) or low-molecular-weight metabolites (LMWM) spectra [7,12,35]. The strength of the NMR metabolomics research platform lies in its ability to simultaneously interrogate disease associations for a large number of metabolites, as well as to assess overall metabolic health in epidemiological studies [7,12,35]. As proposed recently, however, the NMR metabolomics applications, where the spectral data in its entirety is used as the basis for multivariate statistical analyses, are unlikely to provide a reliable basis for providing individual test results in the clinical laboratory setting [36]. In contrast, our deconvolution based NMR assay quantifies BCAA in serum or plasma while taking into account the overlapping signals from the proteins and lipoproteins in each sample, thereby allowing quantification from spectra collected for other clinical purposes, for example quantification of LDL particle number (LDL-P) for management of cardiovascular risk or GlycA for cardiovascular risk assessment [23,37]. Similar to the LDL-P and GlycA tests, the BCAA assay is high-throughput. The time for the assay is only 90 s and there is no need for sample preparation before placing it on the clinical instrument for analysis. With that said, we acknowledge that the %CV for isoleucine, for example, is fairly high because we have sacrificed sensitivity to detect small molecule analytes that circulate at relatively lower concentrations in order to quantify them from spectra that include NMR signals from proteins and lipoproteins. Nonetheless, the analytical performance data revealed that the BCAA assay is robust and suitable for clinical testing. In addition, serum levels of valine, isoleucine and leucine, quantified by NMR, were highly correlated to those measured by mass spectrometry, a platform that is highly sensitive and specific. Therefore, BCAA concentrations obtained using the here described NMR method may be useful for clinical purposes.

Because this assay is novel, we sought to determine if BCAA levels, measured in this way, were associated with clinical outcomes. Consistent with recent literature, analysis from 2 independent cohorts showed that NMR-measured total BCAA, valine, leucine and isoleucine are elevated in subjects with T2DM compared to non-diabetic subjects [3,5–13]. In further analysis total BCAA were independently and positively associated with T2DM and MetS in each cohort. In line, we recently reported that in subjects with varying degrees of glucose tolerance, NMR BCAA levels are closely related to insulin resistance, as assessed by homeostasis model assessment [17]. Although in a small number of participants, our current study also suggested that total BCAA may be directly related to cIMT as a proxy of subclinical atherosclerosis, independent of the presence of T2DM, MetS or its individual components [3,4,10,16]. Though preliminary, this cross-sectional association adds to recent observations showing that BCAA predict incident CVD, in particular stroke [14,16]. Potential mechanisms leading to alterations in circulating BCAA concentrations include excess dietary consumption, increased production by gut microbes, elevated protein degradation and decreased BCAA catabolism in muscle and adipose tissue, due to reduced expression of genes involved in the BCAA catabolic pathway [3,11,15].

Given that BCAA may predict T2DM, BCAA measurement could be useful for determining early metabolic dysfunction, well in advance of the development of chronic disease, thereby enabling preventative measures to potentially reduce disease progression [14,38,39]. To this end, NMR spectroscopy-measured valine, incorporated into a multimarker algorithm, was able to contribute to the prediction of future T2DM [40]. Individual or total BCAA may also be useful in biomarker panels for discerning the metabolic pathways that are altered in specific patient categories, allowing for personalized treatment paradigms. While NMR technology is not prevalent in most clinical laboratories to date, it is available for clinical sample testing in one of the largest clinical laboratories in the US and is becoming more widely available with the dissemination of NMR instruments in multiple laboratories across North America and Europe.

Several methodological issues regarding the present study should be acknowledged. We consider it a strength that the association of total BCAA with T2DM was documented in 2 separate cohorts, i.e. IRAS comprising multiethnic individuals and the Groningen study which was carried out among white subjects from north European descent. Obviously, the cross-sectional design of these studies does neither allow us to address the nature of the observed relationships, nor to exclude the possibility of reverse causation. Thus, it remains possible that BCAA may be elevated as a consequence of metabolic alterations leading to T2DM. Furthermore, subjects using insulin did not participate in either study, making it likely that subjects without severe hyperglycemia were preferentially included. While this may to some extent limit generalizability of our findings, it seems plausible that the absence of insulin using T2DM subjects in our study populations could have resulted in some underestimation with respect to the extent to which elevated BCAA levels are associated with dysglycemia. Finally, in the age- and ex-adjusted multivariable linear regression analysis regarding the independent association of cIMT with BCAA we included both the presence of T2DM and MetS and in alternative analysis the individual MetS components. In each analysis, the total BCAA concentration was associated with cIMT.

In conclusion, BCAA levels can be obtained with sufficient precision and accuracy from the same NMR spectra acquired for lipoprotein

particle concentrations and the GlycA test. NMR-measured BCAA are elevated in MetS and T2DM, and associate with cIMT. Although clinical utility for a standalone BCAA assay has not been established, BCAA quantification in conjunction with other well established markers may be useful for disease detection and prediction, as well as for monitoring disease progression and treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.02.001.

Conflicts interest

JWD, IS, SPM, RPG, RG, JDO and MAC are employees of LabCorp.

Author contributions

JWD, IS, SPM and JDO designed the BCAA assay. JWD and SPM collected the analytical performance data. RPG and RG helped develop the LC-MS/MS BCAA assay and provided the LC-MS/MS data. EGG, SJLB and RPFD designed and analyzed the clinical data; RPFD, MAC and JWD interpreted the data and wrote the manuscript. All authors reviewed the manuscript for accuracy and have approved its submission for publication.

Acknowledgements

The authors would like to thank Brian Rappold for assistance with development of the mass spectrometry based amino acid assays. There are no funding sources to disclose for this study.

References

[1] M.A. Kumar, A.R.R. Bitla, K.V.N. Raju, S.M. Manohar, V.S. Kumar, S.R. Pemmaraju, V.L. Narasimha, Branched chain amino acid profile in early chronic kidney disease, Saudi J. Kidney Disease Transpl. 23 (2012) 1202–1207.
[2] B.C. Batch, K. Hyland, L.P. Svetkey, Branch chain amino acids: biomarkers of health and disease, Curr. Opin. Clin. Nutr. Metab. Care 17 (2014) 86–89.
[3] C.J. Lynch, S.H. Adams, Branched-chain amino acids in metabolic signalling and insulin resistance, Nat. Rev. Endocrinol. 10 (2014) 723–736.
[4] S. Bhattacharya, C.B. Granger, D. Craig, C. Haynes, J. Bain, R.D. Stevens, et al., Validation of the association between a branched chain amino acid metabolite profile and extremes of coronary artery disease in patients referred for cardiac catheterization, Atherosclerosis 232 (2014) 191–196.
[5] C.B. Newgard, J. An, J.R. Bain, M.J. Muehlbauer, R.D. Stevens, L.F. Lien, et al., A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance, Cell Metab. 9 (2009) 311–326.
[6] O. Fiehn, W.T. Garvey, J.W. Newman, K.H. Lok, C.L. Hoppel, S.H. Adams, Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women, PLoS One 5 (2010) e15234.
[7] K. Suhre, C. Meisinger, A. Doring, E. Altmaier, P. Belcredi, C. Gieger, et al., Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting, PLoS One 5 (2010) e13953.
[8] I.R. Lanza, S. Zhang, L.E. Ward, H. Karakelides, D. Raftery, K.S. Nair, Quantitative metabolomics by H-NMR and LC/MS/MS confirms altered metabolic pathways in diabetes, PLoS One 5 (2010) e10538.
[9] T.J. Wang, M.G. Larson, R.S. Vasan, S. Cheng, E.P. Rhee, E. McCabe, et al., Metabolite profiles and the risk of developing diabetes, Nat. Med. 17 (2011) 448–453.
[10] S.H. Shah, J.L. Sun, R.D. Stevens, J.R. Bain, M.J. Muehlbauer, K.S. Pieper, et al., Baseline metabolomic profiles predict cardiovascular events in patients at risk for coronary artery disease, Am. Heart J. 163 (2012) 844–850 (e1).
[11] C.B. Newgard, Interplay between lipids and branched-chain amino acids in development of insulin resistance, Cell Metab. 15 (2012) 606–614.
[12] P. Wurtz, P. Soininen, A.J. Kangas, T. Ronnemaa, T. Lehtimaki, M. Kahonen, et al., Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults, Diabetes Care 36 (2013) 648–655.
[13] A. Floegel, N. Stefan, Z. Yu, K. Muhlenbruch, D. Drogan, H.G. Joost, et al., Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach, Diabetes 62 (2013) 639–648.
[14] M. Magnusson, G.D. Lewis, U. Ericson, M. Orho-Melander, B. Hedblad, G. Engstrom, et al., A diabetes-predictive amino acid score and future cardiovascular disease, Eur. Heart J. 34 (2013) 1982–1989.
[15] H.K. Pedersen, V. Gudmundsdottir, H.B. Nielsen, T. Hyotylainen, T. Nielsen, B.A. Jensen, et al., Human gut microbes impact host serum metabolome and insulin sensitivity, Nature 535 (2016) 376–381.
[16] M. Ruiz-Canela, E. Toledo, C.B. Clish, A. Hruby, L. Liang, J. Salas-Salvado, et al., Plasma branched-chain amino acids and incident cardiovascular disease in the PREDIMED trial, Clin. Chem. 62 (2016) 582–592.
[17] M.A. Connelly, J. Wolak-Dinsmore, R.P.F. Dullaart, Branched chain amino acids are associated with insulin resistance independent of leptin and adiponectin in subjects with varying degrees of glucose tolerance, Metab. Syndr. Relat. Disord. 15 (2017) 183–186.
[18] Y. Mahendran, A. Jonsson, C.T. Have, K.H. Allin, D.R. Witte, M.E. Jorgensen, et al., Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels, Diabetologia 60 (2017) 873–878.
[19] B. Laferrere, D. Reilly, S. Arias, N. Swerdlow, P. Gorroochurn, B. Bawa, et al., Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss, Sci. Transl. Med. 3 (2011) 80re2.
[20] S.H. Shah, D.R. Crosslin, C.S. Haynes, S. Nelson, C.B. Turer, R.D. Stevens, et al., Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss, Diabetologia 55 (2012) 321–330.
[21] R.H. Selzer, H.N. Hodis, H. Kwong-Fu, W.J. Mack, P.L. Lee, C.R. Liu, et al., Evaluation of computerized edge tracking for quantifying intima-media thickness of the common carotid artery from B-mode ultrasound images, Atherosclerosis 111 (1994) 1–11.
[22] D.H. O'Leary, J.F. Polak, R.A. Kronmal, T.A. Manolio, G.L. Burke, S.K. Wolfson Jr., Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study collaborative research group, N. Engl. J. Med. 340 (1999) 14–22.
[23] S.P. Matyus, P.J. Braun, J. Wolak-Dinsmore, E.J. Jeyarajah, I. Shalaurova, Y. Xu, et al., NMR measurement of LDL particle number using the Vantera Clinical Analyzer, Clin. Biochem. 47 (2014) 203–210.
[24] S.P. Matyus, P.J. Braun, J. Wolak-Dinsmore, A.K. Saenger, E.J. Jeyarajah, I. Shalaurova, et al., HDL particle number measured on the Vantera, the first clinical NMR analyzer, Clin. Biochem. 48 (2015) 148–155.
[25] CLSI, Document EP17-a: Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline, Clinical and Laboratory Standards Institute, Wayne, PA USA, 2004.
[26] CLSI, Document EP5-A2: Evaluation of Precision Performance of Quantitative Measurements Methods; Approved Guideline-Second Edition, Clinical and Laboratory Standards Institute, Wayne, PA, 2004.
[27] CLSI, Document EP6-a: Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach, Clinical and Laboratory Standards Institute, Wayne, PA, 2003.
[28] CLSI, Document EP9-A2: Method Comaprison and Bias Estimation Using Patient Samples; Approved Guideline-Second Edition, Clinical and Laboratory Standards Institute, Wayne, PA, 2002.
[29] L.E. Wagenknecht, E.J. Mayer, M. Rewers, S. Haffner, J. Selby, G.M. Borok, et al., The insulin resistance atherosclerosis study (IRAS) objectives, design, and recruitment results, Ann. Epidemiol. 5 (1995) 464–472.
[30] C. Lorenzo, S. Hartnett, A.J. Hanley, M.J. Rewers, L.E. Wagenknecht, A.J. Karter, et al., Impaired fasting glucose and impaired glucose tolerance have distinct lipoprotein and apolipoprotein changes: the insulin resistance atherosclerosis study, J. Clin. Endocrinol. Metab. 98 (2013) 1622–1630.
[31] L. Palaniappan, M.R. Carnethon, Y. Wang, A.J. Hanley, S.P. Fortmann, S.M. Haffner, et al., Predictors of the incident metabolic syndrome in adults: the insulin resistance atherosclerosis study, Diabetes Care 27 (2004) 788–793.
[32] G. Howard, D.H. O'Leary, D. Zaccaro, S. Haffner, M. Rewers, R. Hamman, et al., Insulin sensitivity and atherosclerosis. The insulin resistance atherosclerosis study (IRAS) investigators, Circulation 93 (1996) 1809–1817.
[33] A. Festa, K. Williams, A.J. Hanley, J.D. Otvos, D.C. Goff, L.E. Wagenknecht, et al., Nuclear magnetic resonance lipoprotein abnormalities in prediabetic subjects in the insulin resistance atherosclerosis study, Circulation 111 (2005) 3465–3472.
[34] R. de Vries, F.G. Perton, G.M. Dallinga-Thie, A.M. van Roon, B.H. Wolffenbuttel, A. van Tol, et al., Plasma cholesteryl ester transfer is a determinant of intima-media thickness in type 2 diabetic and nondiabetic subjects: role of CETP and triglycerides, Diabetes 54 (2005) 3554–3559.
[35] P. Soininen, A.J. Kangas, P. Wurtz, T. Suna, M. Ala-Korpela, Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics, Circ. Cardiovasc. Genet. 8 (2015) 192–206.
[36] M. Ala-Korpela, Serum nuclear magnetic resonance spectroscopy: one more step toward clinical utility, Clin. Chem. 61 (2015) 681–683.
[37] J.D. Otvos, I. Shalaurova, J. Wolak-Dinsmore, M.A. Connelly, R.H. Mackey, J.H. Stein, et al., GlycA: a composite nuclear magnetic resonance biomarker of systemic inflammation, Clin. Chem. 61 (2015) 714–723.
[38] K. Nagao, M. Yamakado, The role of amino acid profiles in diabetes risk assessment, Curr Opin Clin Nutr Metab Care 19 (5) (2016) 328–335.
[39] M. Guasch-Ferre, A. Hruby, E. Toledo, C.B. Clish, M.A. Martinez-Gonzalez, J. Salas-Salvado, et al., Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis, Diabetes Care 39 (2016) 833–846.
[40] M.A. Connelly, D.A. Winegar, I. Shalaurova, J.D. Otvos, Nuclear magnetic resonance measured serum biomarkers and type 2 diabetes risk stratification, J. Diab. Metab. Disord. Cont. 2 (4) (2015) 000050.